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## ANNUAL REPORT

### Cachectin/Tumor Necrosis Factor and the Pituitary-Adrenal Axis

ONR Contract No. N00014-88-WM-24020

#### A. Introduction

Cachectin/Tumor Necrosis Factor (TNF) is a macrophage-derived hormone which is released in response to bacterial or parasitic infection and mediates a local inflammatory response. When injected intravenously, TNF produces systemic effects which include metabolic wasting (cachexia), circulatory collapse (shock), and tissue necrosis (see 1 for review). Under conditions when TNF is known to be elevated (septic shock, malignancy, chronic illness), significant endocrine abnormalities also exist. These include an elevation in "stress hormones" and changes in pituitary-thyroid hormones referred to as the "sick euthyroid syndrome". It is unknown whether the observed changes in hormones are a direct effect of TNF or are secondary to the cardiovascular and metabolic effects induced by TNF.

Several lines of evidence indicate that the immune system by way of macrophage cytokines can influence neuroendocrine function and vice versa suggesting that these two systems are involved in a complete regulatory feedback loop (see 2). For example, interleukin-1, a macrophage product released in response to infection, has been shown to directly activate the hypothalamo-pituitary axis resulting in the increased production of ACTH and glucocorticoids (3,4). Glucocorticoids modulate immune and inflammatory responses and have been shown to suppress the synthesis and release of TNF and interleukin-1 in response to endotoxin (5,6). Furthermore, adrenal-cortical hormones (e.g. cortisol) are substantially elevated during chronic illness or stress (7) and possibly contribute to the immunosuppression associated with chronic illness and malignancy. Recently, it has been reported that cortisol levels are elevated following TNF injection in anesthetized dogs (8). However, it is unknown from this study whether the rise in cortisol is due to TNF's direct effect on the adrenal gland, is mediated through TNF-induced ACTH release or is secondary to TNF-induced hypotension and/or other systemic effects. To date, little is known about the interactions between TNF and the pituitary-adrenal axis. In addition, the physiologic regulation of TNF in health or disease remains unclear.

The purpose of our studies was to further explore the interactions between the immune system and the neuroendocrine system. Specifically, we chose to determine what effect TNF has on pituitary ACTH and adrenal corticosterone release both in vitro and in vivo, and conversely, what effect ACTH and glucocorticoids have on the release of TNF from macrophages in response to endotoxin. It is anticipated that results from these studies will provide insight into how the immune system by way of macrophage-derived peptides may regulate endocrine function during infectious challenge, and conversely, how pituitary-adrenal hormones activated during infection or stress may act to regulate the release of TNF and thus modulate immune function.

## B. Research Results

The first year of this project was largely dedicated to in vitro studies because of the initial limited supply (0.5 mg) of recombinant human TNF-alpha obtained from Genentech, Inc. (S. San Francisco, CA) in September 1988. Since September, we have tested the effects of TNF on basal and stimulated corticosterone release from cultured adrenal cells and have observed some novel and exciting findings (presented below). Our fortunate access to human thyroid cells also enabled us to test the effects of TNF on basal and stimulated thyroglobulin release (findings presented below).

We received 2.0 mg of TNF from Genentech in February 1989 and began testing the effects of TNF on ACTH and corticosterone release in vivo. We were also able to monitor the hemodynamic effects of TNF injections in these animals. The uniqueness of these in vivo studies is that they were performed in unanesthetized animals unlike the previously reported studies which were performed in anesthetized animals. Using this model we were able to show significant changes in ACTH and corticosterone which may have been otherwise masked by anesthesia (see below).

We have hired a technician who has learned the techniques for harvesting and culturing murine macrophages and has diligently set up and standardized a TNF bioassay for the purposes of measuring TNF release from cultured macrophages. We are now beginning the final phase of our project which involves testing the effects of ACTH and glucocorticoids on TNF release from activated macrophages.

### 1. Effects of recombinant human TNF-alpha on basal and stimulated corticosterone release from cultured adrenal cells.

Rat adrenal cells were prepared by digestion with collagenase. Cells were then incubated for 2 hours with or without ACTH and TNF alone or in combination. Data of representative experiments are shown in Table 1. TNF alone had no effect on baseline corticosterone release even at the highest dose tested, however, TNF clearly inhibited ACTH stimulated corticosterone secretion. This inhibition is reproducible and consistent and is seen at concentrations of TNF similar to that reported in patients with sepsis and with AIDS. There was no difference in cell number or viability following TNF application with or without ACTH present, indicating that the inhibitory effects of TNF are not due to cytotoxicity. This finding represents a significant and new interaction between the immune and endocrine systems.

### 2. Effects of recombinant human TNF-alpha on basal and stimulated thyroglobulin and cyclic AMP from cultured thyroid cells.

Based on our observation of TNF's inhibitory actions on adrenal cells, we explored the effects of TNF on human thyroid cells. We reasoned that the inhibitory effects of TNF seen with stimulated adrenal cells may extend to other endocrine cells (thyroid gland) which are stimulated by a peptide hormone (TSH). The clinical observation that TNF is elevated during medical conditions that are associated with the "sick euthyroid syndrome" (where the thyroid gland's response to TSH is suppressed) further supports this hypothesis.



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Human thyroid cells were prepared from tissue obtained at thyroideectomy for multimodular goiter or for Graves disease. Cells were digested with collagenase and placed in cultures. After 48 hours cells were trypsinized and replated at a concentration of  $5 \times 10^5$  cells/0.5 cc in multiwell culture plates. After 72 hours media was removed and cells were incubated with TNF at 0, 100, 300 or 1000 pg/ml with or without TSH (1 uU/ml) using previously reported techniques. Media was removed and analyzed for cAMP 2 hours after incubation or after 24 and 48 hours for thyroglobulin measurements. At the end of 48 hours cells were trypsinized and counted. No difference in cell number or viability was noted with TNF with or without TSH. Data from these experiments is shown in Tables 2 and 3.

In summary, TNF inhibited TSH stimulated thyroglobulin secretion in human thyroid cells in a dose-dependent manner. In all experiments, TSH exposure resulted in a brisk increase in cAMP production. However, even at the highest concentration, TNF had no effect on TSH stimulated cAMP production. This suggests that TNF's inhibition of TSH-stimulated thyroglobulin secretion is not mediated through cAMP. It is tempting to speculate that the observed in vitro inhibitory effects of TNF on the thyroid gland may occur in clinical situations (e.g. sick euthyroid syndrome) where TNF levels are increased and thyroid function is suppressed.

### 3. Effects of recombinant human TNF-alpha on plasma levels of ACTH and corticosterone in the unanesthetized rat.

We initially experienced some challenges in developing an in vivo model that would accurately assess the effects of TNF on ACTH and corticosterone release. It is known, for instance, that surgical stress, anesthesia, and repeated bleedings can induce significant changes in ACTH and corticosterone release themselves. Therefore, we chose to perform surgeries the day before the experiment, to limit blood sampling times and volumes to a minimum, and to immediately replace withdrawn blood with an equal volume of donor rat blood. Donor blood was obtained by cardiac puncture from donor rats on the morning of the experiment. ACTH and corticosterone levels in donor blood was found to be well within the normal range ( $68 \pm 12$  pg/ml). Experiments were always run between the hours of 1000 and 1300 to control for the effects of diurnal variation on plasma hormone levels.

TNF was made up fresh on the morning of the experiment at doses of 0.01, 0.03 and 0.10 mg/ml and injected (1 ml/kg) as an intravenous bolus. Control animals received bovine serum albumin (BSA; 2 mg/kg) as a bolus injection (1 ml/kg). One ml of venous blood was withdrawn before and at 15, 30, 45, 60, and 120 minutes after TNF injection. Plasma ACTH and corticosterone levels were assayed using commercially available kits. Preliminary data (n=3-6) is presented in Figure 1 and 2.

In summary, TNF at all three doses induced significant increases in circulating ACTH and corticosterone levels. Interestingly, TNF at a dose of 0.01 mg/kg induced as great a response in ACTH as the 0.10 mg/kg TNF dose. As expected, corticosterone levels were subsequently elevated in these animals. Based on the in vitro observation that TNF inhibits ACTH-induced corticosterone release, it would be interesting to determine whether these inhibitory effects are also present in vivo. Unfortunately, the present in vivo model did not allow us to test this hypothesis.

4. Effects of recombinant human TNF-alpha on mean arterial pressure, heart rate and pulse pressure in the unanesthetized rat.

On the day of surgery, an arterial catheter was implanted for the purpose of cardiovascular monitoring. On the experimental day, hemodynamic responses (mean arterial pressure, heart rate and pulse pressure) to TNF injections were monitored using a physiograph (Buxco Electronics).

Preliminary data ( $n=2-6$ ) is presented in Figures 3, 4, and 5. Due to the small number of animals per group, it was difficult to assess statistical differences between treatment groups. The only notable and consistent response to TNF injection was an increase in heart rate at 5-10 minutes post-injection. There were no differences in mean arterial pressure within or between treatment groups. At the higher doses of TNF (0.03 and 0.10 mg/kg), pulse pressure tended to fall after 60 minutes. It should also be noted that blood withdrawal (from the venous line) occurred at repeated intervals during the time of cardiovascular monitoring and may have contributed to some of the observed fluctuations in hemodynamics.

Considering the small doses of TNF used, it is not surprising that TNF produced little or no change in hemodynamics. Tracey et al. (9) have reported hypotension and tachycardia in anesthetized rats following TNF injection but at higher doses (1.8 mg/kg). Others have reported that TNF infusions into tumor-bearing humans results in a tachycardia (without changes in blood pressure) throughout the period of infusion (10). We likewise have observed a significant and reproducible tachycardia. Since this increase in heart rate is present in the absence of significant hypotension, this suggests that TNF induced tachycardia may be mediated directly at the level of the heart or through some other systemic effect of TNF.

c. **SUMMARY**

Preliminary studies investigating immune-neuroendocrine interactions have yielded several interesting and novel findings. Intravenous injections of low doses of TNF (0.01-0.1 mg/kg) in unanesthetized animals resulted in significant elevations in circulating ACTH and corticosterone. These low doses of TNF did not produce changes in mean arterial pressure but did significantly increase heart rate within 5-10 minutes post-injection. This is the first report of TNF's effects on ACTH and corticosterone release in unanesthetized animals.

In vitro TNF was without effect on the basal secretion of corticosterone from adrenal cells, however, TNF potently inhibited ACTH-stimulated corticosterone release. Similarly, TNF was shown to inhibit TSH-stimulated thyroglobulin release from cultured thyroid cells without affecting the basal secretion of this hormone. These findings represent a significant and new interaction between the immune and endocrine systems. Furthermore, the remarkable inhibitory effect of TNF on stimulated adrenal and thyroid hormone release may have relevance in clinical situations when TNF levels are high (e.g. critical illness, malignancy). Particularly, it is interesting to speculate that the observed inhibition of stimulated thyroid hormone release may be associated with the development of the "sick euthyroid syndrome" during critical illness.

**d. PUBLICATIONS**

Poth, M., Tseng, Y.L., Wartofsky, L. Does cachectin mediate altered thyroid function in systemic illness? A cell culture model. To be presented at the 1989 Endocrine Society meeting.

Brennan, M.J., Betz, J.A., Poth, M. Tumor Necrosis Factor inhibits ACTH stimulated corticosterone secretion by rat adrenal cortical cells. To be presented at the 1989 Endocrine Society meeting.

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3. Bernton EW, Beach JE, Holaday JW, et al. Science 238:519-521, 1987.
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8. Tracey KJ, Lowry SF, Fahey TJ, et al. Surg, Gyn & Obs 164:415-422, 1987.
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**TABLE 1.** Effects of TNF on stimulated corticosterone release

Corticosterone secreted (ng/well)			
<u>ACTH</u>	<u>TNF (ug/ml)</u>	<u>Experiment I</u>	<u>Experiment II</u>
0	0	14 $\pm$ 2	6.4 $\pm$ 1.2
30	0	41.7 $\pm$ 7.8	16.9 $\pm$ 5.3
100	0	77.3 $\pm$ 6.2	63.0 $\pm$ 2.0
<hr/>			
30	100	2.6 $\pm$ .20	8.1 $\pm$ 5.3
30	300	.28 $\pm$ .03	
30	1000	.31 $\pm$ .02	3.6 $\pm$ .20
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100	100	1.21 $\pm$ .17	10.9 $\pm$ 1.7
100	300	3.98 $\pm$ 3.0	
100	1000	3.70 $\pm$ 2.15	3.5 $\pm$ 0
<hr/>			

Numbers are means of 3 wells

**TABLE 2.** Effects of TNF on basal and stimulated thyroglobulin release

Experiment I Thyroglobulin (ng/well) at 0-24 hours

	TNF (pg/ml)			
	0	100	300	1000
Without TSH	70 <u>±</u> 6	79 <u>±</u> 3	73 <u>±</u> 1	65 <u>±</u> 5
With TSH	340 <u>±</u> 35	212 <u>±</u> 25*	171 <u>±</u> 17*	65 <u>±</u> 21*

Experiment I Thyroglobulin (ng/well) at 24-48 hours

	TNF (pg/ml)			
	0	100	300	1000
Without TSH	114 <u>±</u> 33	94 <u>±</u> 10	79 <u>±</u> 8	56 <u>±</u> 12
With TSH	630 <u>±</u> 126	251 <u>±</u> 25*	160 <u>±</u> 23*	63 <u>±</u> 10*

Data is mean ± S.D. using data from 3 wells

\*Different from control (TNF=0) with p<0.01

Experiment II Thyroglobulin (ng/well) at 24-48 hours

	TNF (pg/ml)			
	0	100	300	1000
Without TSH	369 <u>±</u> 19	334 <u>±</u> 5	258 <u>±</u> 16*	223 <u>±</u> 36*
With TSH	1025 <u>±</u> 15	704 <u>±</u> 44*	285 <u>±</u> 51*	105 <u>±</u> 11*

\*Data significantly different from control (TNF=0) according to ANOVA t-test (p<0.05).

**TABLE 3.** Effects of TNF on TSH-induced cAMP release

		<u>cAMP (picomoles/well/2 hours)</u>		
		TNF (pg/ml)		
		0	100	300
<b>Without TSH</b>		<0.5	<0.5	<0.5
<b>With TSH</b>		5.6	5.0	4.7

Data are Mean of 3 wells.

Figure 1. Effects of TNF on plasma levels of ACTH

# ACTH

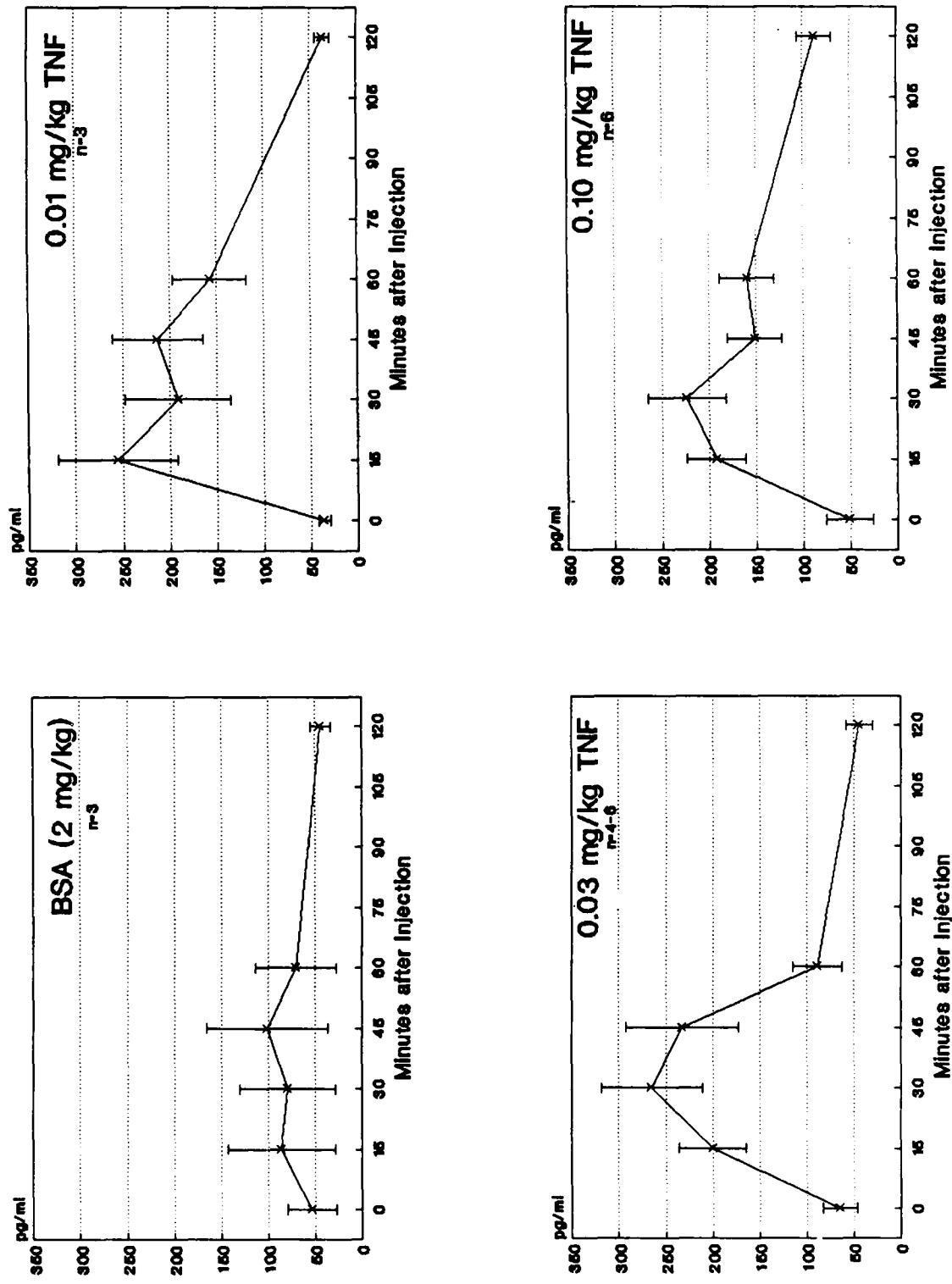


Figure 2. Effects of TNF on plasma levels of corticosterone

# CORTICOSTERONE

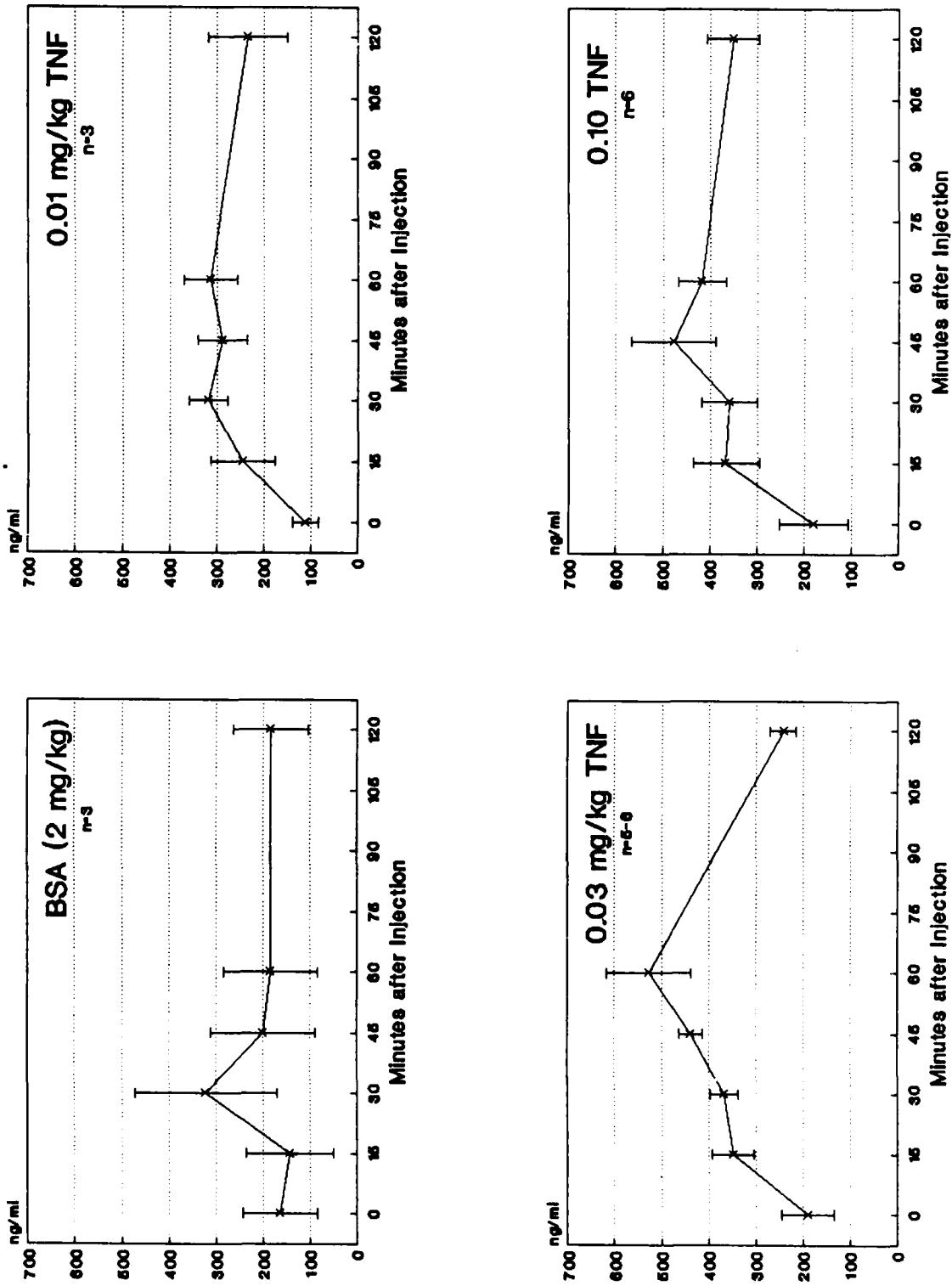


Figure 3. Effects of TNF on mean arterial pressure

# MEAN ARTERIAL PRESSURE

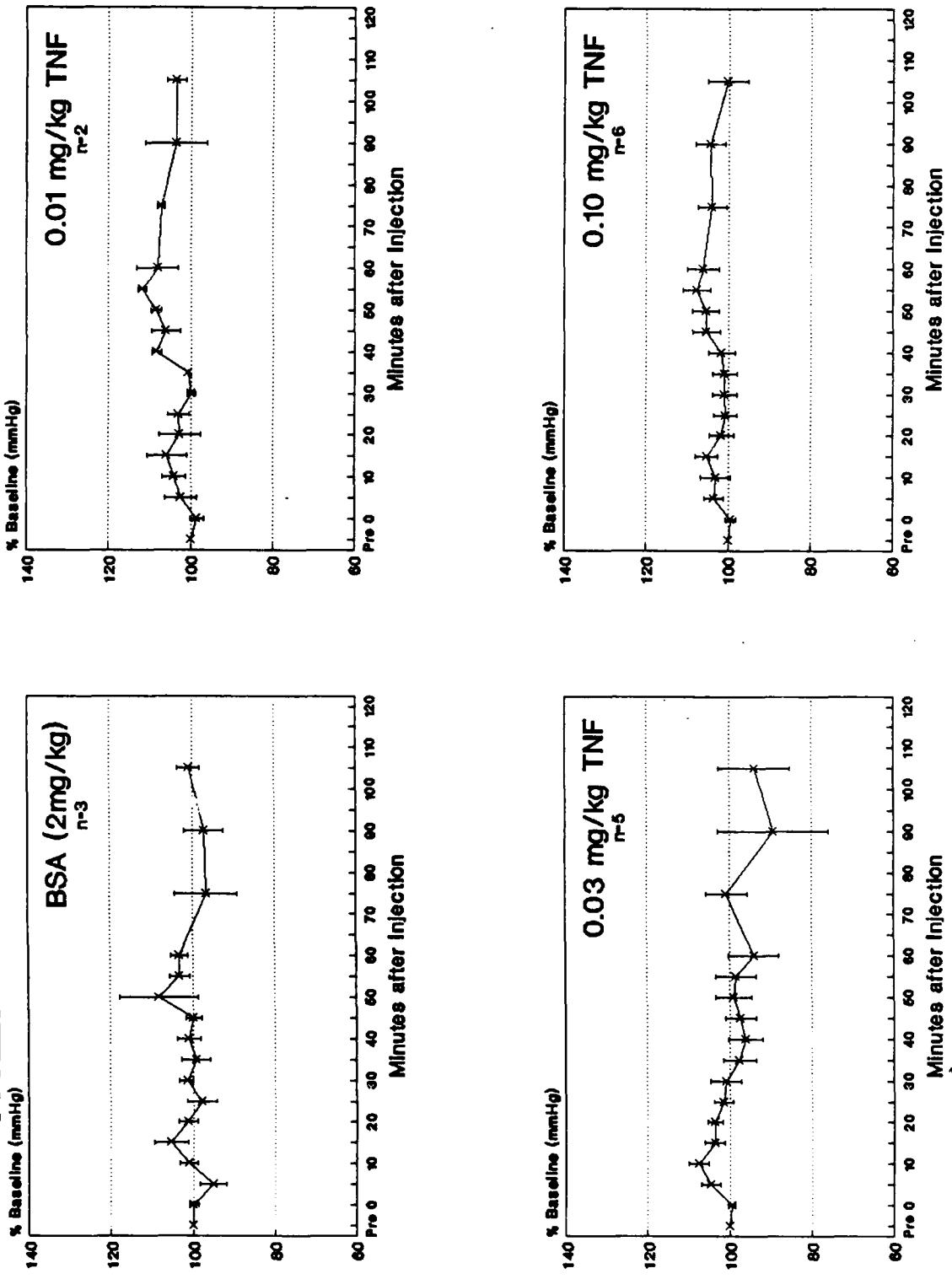


Figure 4. Effects of TNF on heart rate

# HEART RATE

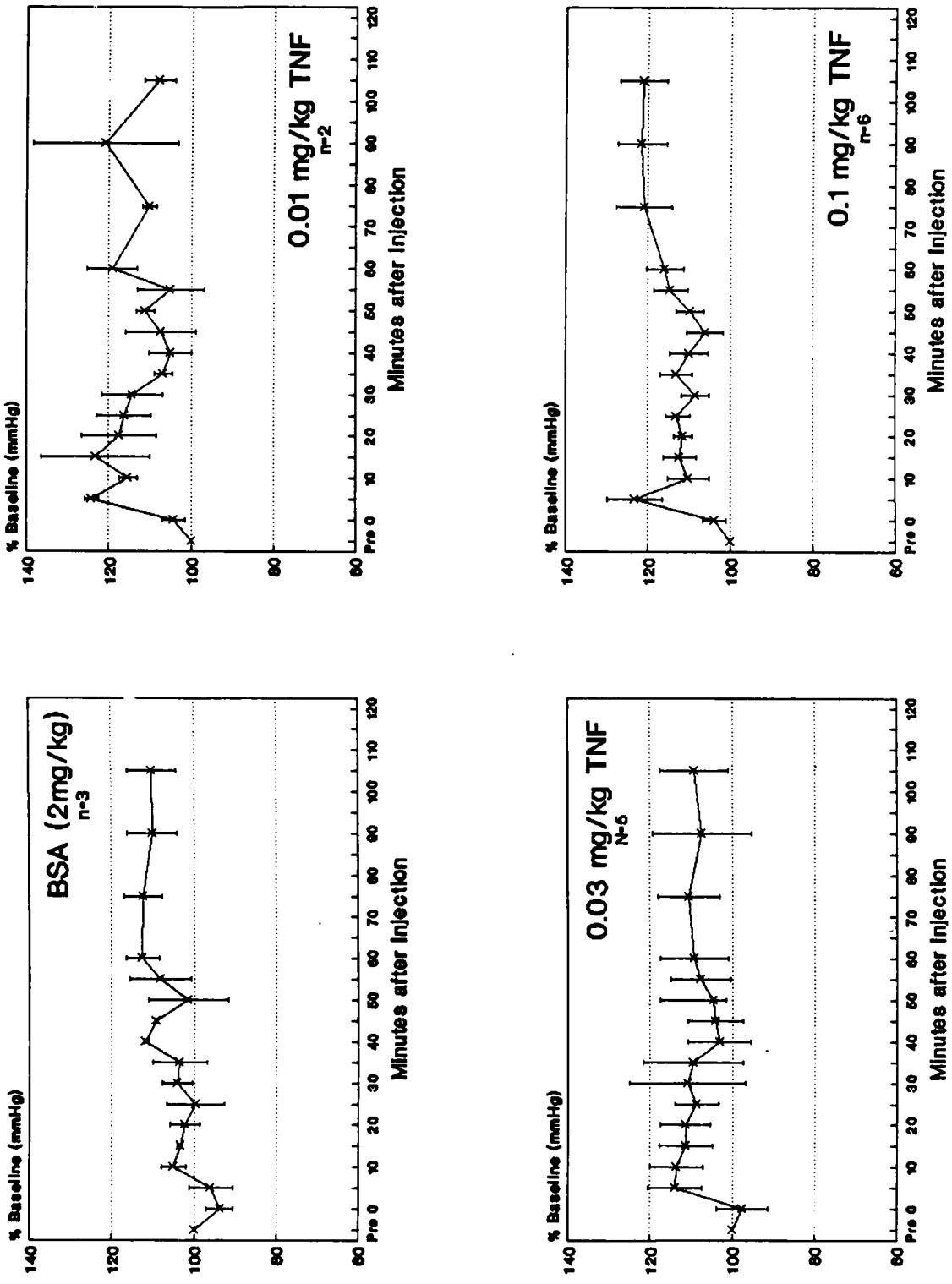
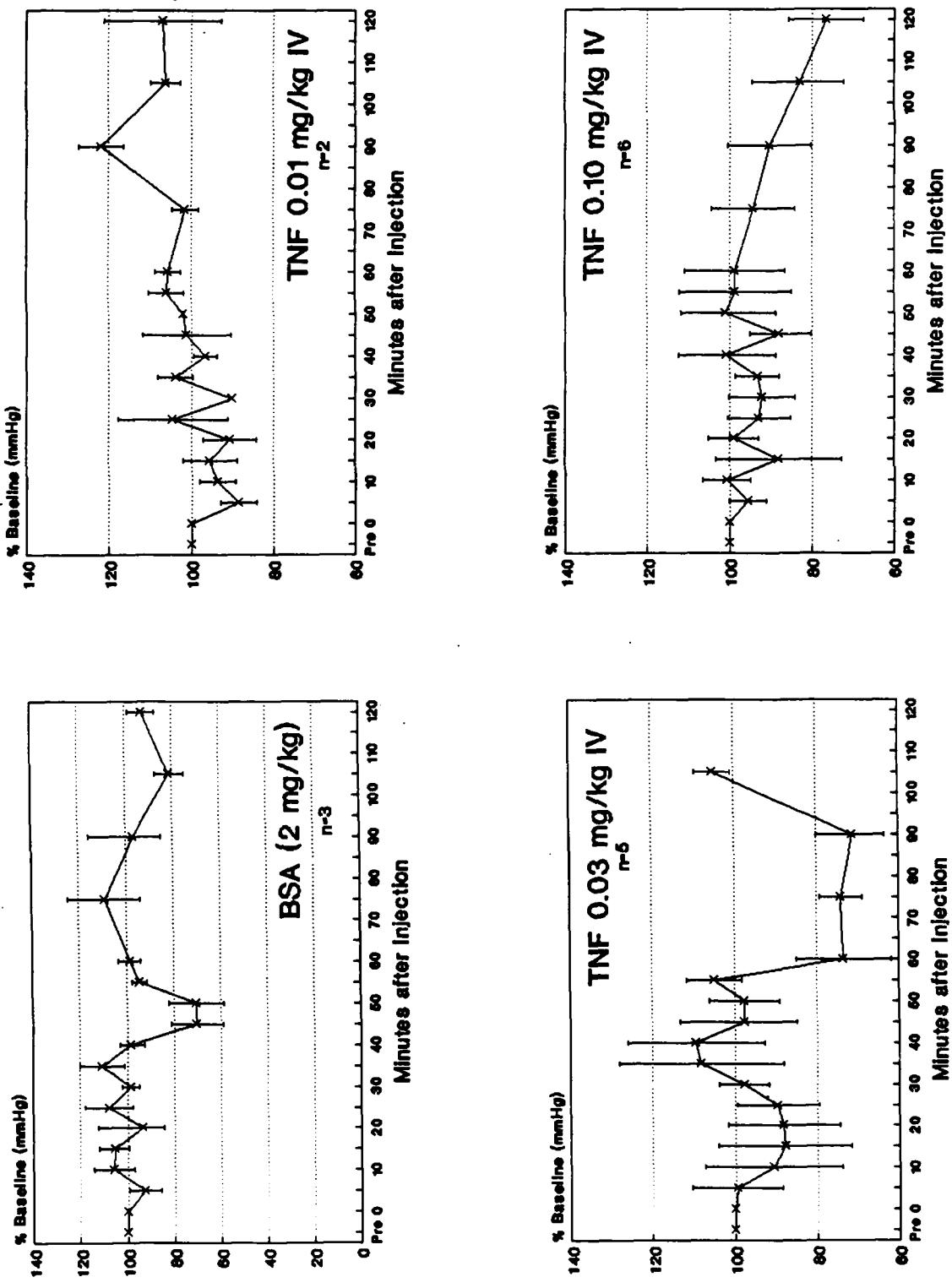


Figure 5. Effects of TNF on pulse pressure

# PULSE PRESSURE



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DOES CACHECTIN MEDIATE ALTERED THYROID FUNCTION IN SYSTEMIC ILLNESS? A CELL CULTURE MODEL. M. Poth, Y.L. Tseng\*, and L. Wartofsky. Walter Reed Army Medical Center, Washington, DC 20307 and Uniformed Services University of the Health Sciences, Bethesda, MD 20814

Thyroidal economy in systemic non-thyroidal illness (SNTI) is marked by reductions in both central thyroid function and peripheral  $T_4$  to  $T_3$  conversion presumed to reflect a homeostatic mechanism to conserve energy. TSH levels tend to be normal in SNTI, and the mechanism underlying reduced thyroidal secretion is unknown. Recently, Ozawa (Endocrinol 123:1461, 1988) treated mice with tumor necrosis factor (TNF), as an animal model for SNTI, and reported diminished  $T_3$  and  $T_4$  responses to TSH administration. We have employed a primary thyroid cell culture system derived from surgical specimens to assess the effects of TNF on thyroidal responses to TSH. Cells at a density of 100,000/well were incubated with various concentrations (0-1000 pg/ml) of recombinant alpha-TNF (Genentech) and bTSH (1 mU/ml). Media were analyzed for cyclic AMP by RIA, and for thyroglobulin (Tg) by ELISA. TNF had no effect on either basal or TSH-stimulated cAMP generation.

Tg (ng/well) secreted into media by thyroid cells (100,000 cells/well) in the presence of TNF and bTSH (Mean  $\pm$  SEM)

TNF (pg/ml)	0-24 hr		24-48 hr	
	(-) TSH	(+) TSH	(-) TSH	(+) TSH
0	212 $\pm$ 7	365 $\pm$ 56	369 $\pm$ 19	1025 $\pm$ 15
100	186 $\pm$ 23	266 $\pm$ 57	334 $\pm$ 5	704 $\pm$ 44*
300	266 $\pm$ 73	144 $\pm$ 23*	258 $\pm$ 16*	285 $\pm$ 51*
1000	240 $\pm$ 41	89 $\pm$ 12*	223 $\pm$ 36*	105 $\pm$ 11*

\*Data significantly different from control (TNF=0) according to ANOVA t-test ( $p < 0.05$ ).

While TNF alone had no effect on Tg release at 24 hrs, TNF blunted TSH-stimulated Tg release by 27-76%. At 48 hrs, TNF blunted Tg release by 9-39% and TSH-stimulated Tg release by 31-90%. These results are consistent with the *in vivo* observations of Ozawa et al. and demonstrate a cytostatic effect of human thyrocytes by TNF in concentrations comparable to blood levels in man during SNTI. Thus, increases in circulating TNF in SNTI may be responsible for reduced thyroid function in these patients.

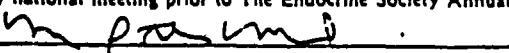
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TUMOR NECROSIS FACTOR INHIBITS ACTH STIMULATED CORTICOSTERONE SECRETION BY RAT ADRENAL CORTICAL CELLS. M.J. Brennan\*, J.A. Betz\*, and M. Poth. (SPON: D. Bunner) Walter Reed Army Medical Center, Washington, D.C. 20307 and The Uniformed Services University of the Health Sciences, Bethesda, M.D. 20814.

Tumor Necrosis factor (TNF) is a biologically active peptide secreted by macrophages and monocytes. TNF secretion is stimulated by endotoxin and TNF has been implicated in the pathogenesis of septic shock. To determine if TNF has specific actions on the adrenal gland, we studied the effects of ACTH and TNF on the *in vitro* secretion of corticosterone by rat adrenal cells. Adrenal glands from adult Sprague-Dawley rats were harvested, digested with collagenase, and cell suspensions were prepared. Cell cultures were incubated for 90 minutes in media with various concentrations of ACTH, TNF, or ACTH and TNF in combination. The cells were then centrifuged and the supernatants were assayed by RIA for corticosterone.

### Results:

### Corticosterone Secretion (pg/400,000 cells)

ACTH (pg/ml)	TNF (pg/ml)		
	0	100	1000
0	6.4 + 1.2	5.8 + 1.2	4.4 + .20
30	16.9 + 5.3	8.1 + 5.3	3.6 + .20
100	63.0 + 2.0	10.9 + 1.7	3.5 + .00

Incubation with ACTH at concentrations of 10, 30 and 100 pg/ml produced a dose-response related stimulation of corticosterone secretion. Incubation with TNF alone at concentrations of 100, 300, and 1000 pg/ml had no effect on corticosterone secretion. However, when adrenal cells were incubated with ACTH and TNF in combination, corticosterone secretion was significantly inhibited. TNF at 100 pg/ml inhibited ACTH stimulated corticosterone secretion by 75-100% ( $p < .001$ ), while TNF at 1000 pg/ml produced 100% inhibition ( $p < .001$ ). Statistical significance was determined by multiple regression analysis. Conclusion: TNF inhibits ACTH stimulation of corticosterone secretion by rat adrenocortical cells. This is a new, potentially clinically important interaction between the immune and endocrine systems. Speculation: TNF may potentiate septic shock by inhibiting the body's ability to mount an appropriate glucocorticoid response to the stress of sepsis.

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